Microscopy Today
Volume 2-00
January/February 2000
Pages 26-32

NOVEL COMBINATION OF FLUORESCENCE MICROSCOPY WITH INFRARED MICRO-SPECTROSCOPY

Lisa Miller (BNL-NSLS) and Tom Tague (Spectra Tech, Inc.)

Infrared micro-spectroscopy (IRMS) is a widely used and valuable technique for identifying the chemical makeup of small particles. By taking advantage of the high brightness of a synchrotron infrared source, particles that are too tiny to be analyzed with a conventional thermal (globar) source can now be examined in detail.[1,2] With the advent of the synchrotron infrared microscope, new applications of IRMS are just beginning to be realized.[3-5] Frequently, sample visualization is benefited by the use of fluorescence illumination. Fluorescent compounds absorb light and then emit light at wavelengths longer than the absorbed wavelength. Primary (natural) fluorescence is known to occur in plant cell walls, wool, as well as many pharmaceutical products. Secondary fluorescence is the use of fluorescent dyes or fluorochromes to illuminate samples which do not exhibit native fluorescence. The dyes typically are bound to the compounds of interest. Fluorescence is particularly useful for visualizing samples against a dark background and can have a detectivity of 50 molecules per square micron. By the 1950's, the use of secondary fluorescence had made fluorescence microscopy a common analytical tool.

To date, combining fluorescence microscopy and infrared microspectroscopy has required analyses with two separate microscopes. This procedure required the user to somehow mark the fluorescent regions of interest (often with a photograph) before transferring the sample to the infrared microscope. The optical design (finite tube length) of earlier infrared microscopes did not allow the ready insertion of accessory optics, such as optical components for fluorescence microscopy. For the first time, an infrared microscope has been modified such that fluorescence sample visualization and infrared microspectroscopic analysis can be performed simultaneously. This novel infrared microscope, located at Beamline U10B, has applicability to many fields of investigation, such as biophysics, cell biology, medicine, forensics, microelectronics, and geology.

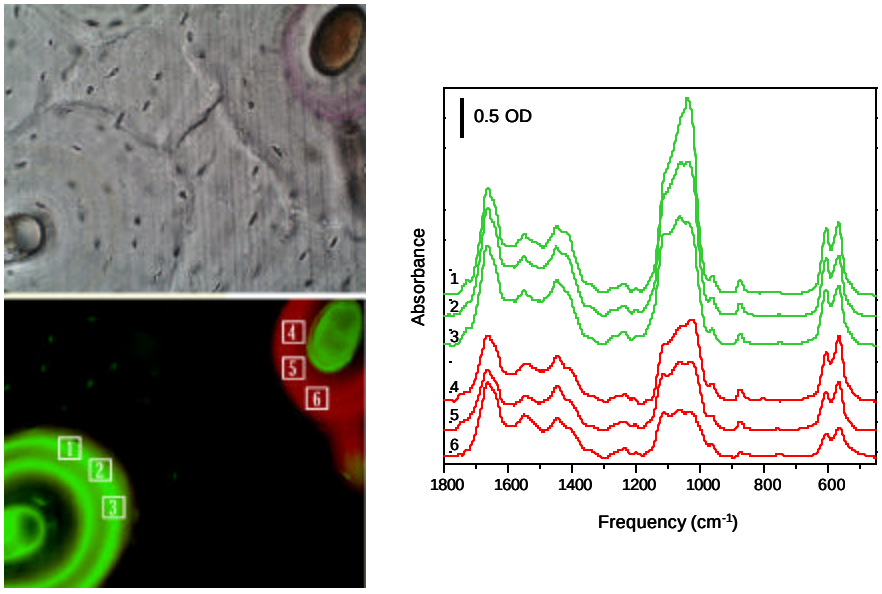

Figure 1. *Optical images of a region of cortical bone from an Ovx monkey tibia under **(top left)** normal transmission illumination and **(bottom left)** ultraviolet fluorescence illumination. The bone sample is embedded in poly- methyl methacrylate and microtomed to a 5 mm thickness. **(Right)** Individual IR spectra were collected in the calcein-labeled region of bone (spectra 1-3) and alizarin complexone-labeled region of bone (spectra 4-6). For each of these spectra, 128 scans were collected in transmission mode at 4 cm^{-1} resolution using a 6 x 6 mm square aperture and a Cu-doped Ge detector.*

Biomedical studies

It is becoming increasing clear that IRMS is a powerful tool for determining the chemical composition of biological and biomedical samples. This is largely due to the dramatic im-

provement in spatial resolution attainable using a synchrotron infrared source. The high brightness of the synchrotron source permits high quality data collection at the diffraction limit, e.g. 3-25 μm spatial resolution in the mid-infrared region (4000 – 400 cm^{-1}). In contrast, fluorescence illumination has been a commonly used technique in biology for many years. Immunofluorescence, where fluorochrome labels are attached to specific antibodies, has become a widespread and powerful technique in cell biology for visualizing targeted antigens.

One important use of fluorochrome labels has been in the study of osteoporosis. Throughout a lifetime, bone is remodeled, i.e. old bone is eroded away and new bone is deposited. Although it is clear that osteoporosis is associated with a reduction in bone mass and a fragile skeleton, it is not understood whether the chemical composition of bone remodeled after the onset of osteoporosis is different from normal bone. Changes in the bone chemistry associated with osteoporosis might reveal important information pertaining to the cause of osteoporosis and how it might be treated.

Female monkeys *(Macaca fascicularis)* that have had their ovaries removed often develop osteoporosis, similar to postmenopausal women. In studies by Jerome and coworkers, a colony of these monkeys were administered fluorochrome labels at one and two years after ovariectomy (Ovx) or Sham ovariectomy (Intact).[6] These labels were taken up into newly remodeled bone (Figure 1). Green (calcein fluorochrome) and orange (alizarin complexone fluorochrome) fluorescence represent new bone deposited one and two years after surgery, respectively. By combining fluorescence microscopy and IRMS, the chemical composition of newly remodeled bone from Intact versus Ovx monkeys has been compared.[7,8] Figure 1 shows infrared spectra of bone remodeled 1 year (spectra 1-3) and 2 years (spectra 4-6) after ovariectomy. Infrared absorption bands present at 500-650 and 900-1200 cm^{-1} ($-PO_4^{-3}$) and 850-880 cm^{-1} ($-CO_3^{-2}$) indicate the presence of mineral in the bone. Peaks at 1660 and 1550 cm^{-1} reveal Amide I and Amide II absorption bands, respectively, indicating the presence of protein (primarily collagen) in the bone. There are no infrared absorption bands present in the spectrum resulting from the fluorescent dyes. Secondary fluorescing dyes are typically utilized at concentrations dilute enough to be undetectable in the infrared region.

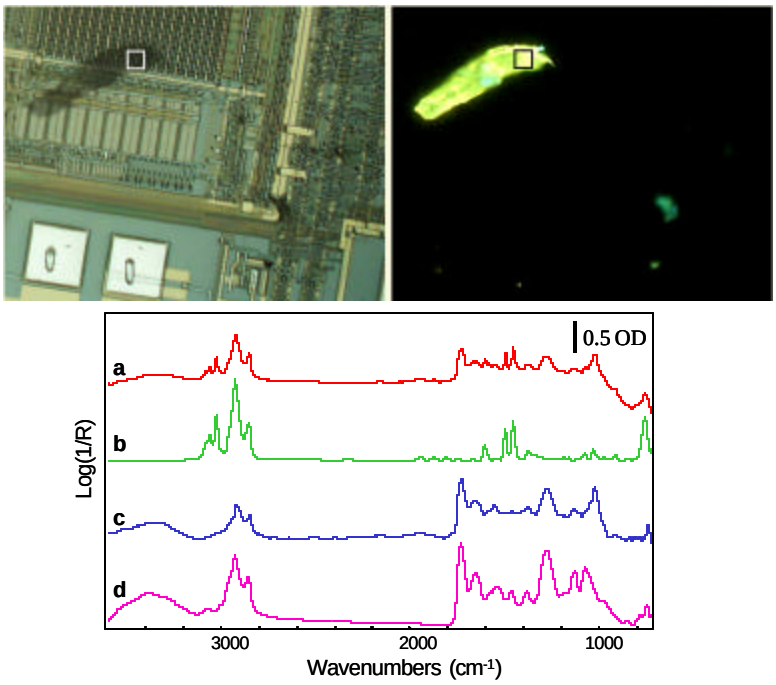

Figure 2. *Optical images of an integrated wafer under* ***(top left)*** *normal reflection illumination, and* ***(top right)*** *ultraviolet fluorescence illumination.* ***(Bottom)*** *Infrared spectra collected from* ***(a)*** *the highlighted particle on the integrated wafer, and* ***(b)*** *an 800 MW polystyrene from the Hummel Polymer Reference Library (Nicolet Instrument Corp.).* ***(c)*** *Infrared spectrum resulting from the subtraction of the polystyrene reference spectrum from the raw spectrum.* ***(d)*** *Infrared spectrum of an alkyd urea resin, also from the Hummel Polymer Library. For each of the IR spectrum of the contaminant, 128 scans were collected in reflection mode at 4 cm^{-1} resolution using a 10 x10 mm square aperture and an MCT-A detector.*

Comparison of the fluorochrome-labeled bone in Intact versus Ovx monkeys indicates that bone from monkeys with osteoporosis can be characterized as having abnormal collagen structure and reduced rates of mineralization. Coupled with factors such as trabecular architecture and bone shape and size, these ultrastructural factors may play a contributing role in the increased bone fragility in osteoporosis.

Microelectronics

The microelectronics industry continues to be focused on miniaturization of integrated components on silicon wafers. For the integration of microcomponents to be effective, the wafer must be clean to the parts per billion level.[9] After component integration takes place, individual components can suffer damage when conducting surface contaminants are present. Typically, quality control is performed at many steps in the manufacturing process to ensure that the product is free of contaminants. When contaminants are found, it is vital that chemical characterization of the particles takes place to determine the source of contamination.

Currently, there are no satisfactory techniques for performing quality control on semiconductor wafers and circuit boards. Typically, an optical microscope is used to scan for contaminant particles. This can be a tedious process and is particularly difficult when investigating integrated circuits. Under normal illumination, it is difficult to discern the presence of dust and other small contaminant particles within an integrated circuit (Figure 2). In contrast, fluorescence illumination with ultraviolet light cloaks the non-fluorescing components, making it easy to identify contaminant particles. Once identified, an aperture is set to mask a specific particle and IRMS is used to characterize the chemical makeup of that contaminant.[10,11] Infrared analysis of one of the contaminant particles in Figure 2 reveals the presence of low molecular weight polystyrene (Figure 2a). Figure 2b shows a reference spectrum of polystyrene (molecular weight 800) and 2c shows the result of mathematically subtracting the polystyrene spectrum from the raw spectrum. Finally, Figure 2d shows a reference spectrum of an alkyd urea resin, which closely matches the subtraction result found in Figure 2c. Thus, the identification of the these contaminants allows for precise identification of their origins in the manufacturing process.

Beamline U10B

Beginning in May 2000, Beamline U10B will be available for General Users. The beamline will be equipped with a Nicolet Magna 860 step-scan FTIR and Spectra Tech Continuμm infrared microscope (Figure 3). Beamsplitters and detectors are available for the mid- and far-infrared regions. For fluorescence microscopy, the excitation source is a mercury arc lamp, where wavelengths are selected by utilizing Olympus fluorescence cubes. In addition to fluorescence imaging capabilities, sample viewing can also be enhanced with visible light polarizers and differential interference contrast (DIC).

Figure 3. *Gywn Williams (NSLS) at the helm of the new Spectra Tech Continuμm infrared microscope with fluorescence microscopy capabilities.*

The Continuμm infrared microscope is the first commercial system that has the capability of simultaneous sample viewing and infrared data collection. This feature is especially important for the high-resolution applications that require a synchrotron source, where accurate sample masking is critical. Moreover, this feature saves time for the user by eliminating the need to switch from "view mode" to "infrared mode" for data collection. An automated mapping stage is also a time-saving feature of this system. Software control of the sample position, aperture settings, and mapping simplify the user interface. This ability to rapidly visualize and chemically characterize minute samples provides a new and important capability for a wide range of applications. For more information on Beamline U10B, please contact Lisa Miller at lmiller@bnl.gov.

REFERENCES

1) Carr, G. L.; Reffner, J. A.; Williams, G. P. *Rev. Sci. Instr.* **1995**, *66*, 1490-1492.
2) Reffner, J. A.; Martoglio, P. A.; Williams, G. P. *Rev. Sci. Instr.* **1995**, *66*, 1298.
3) Jamin, N.; Dumas, P.; Moncuit, J.; Fridman, W. H.; Teillaud, J. L.; Carr, G. L.; Williams, G. P. *Proc Natl Acad Sci U S A* **1998**, *95*, 4837-40.
4) Miller, L. M.; Carlson, C. S.; Carr, G. L.; Chance, M. R. *Cell. and Mol. Biol.* **1998**, *44*, 117-127.
5) Wetzel, D. L.; LeVine, S. M. *Science* **1999**, *285*, 1224-1225.
6) Jerome, C. P.; Power, R. A.; Obasanjo, I. O.; Register, T. C.; Guidry, M.; Carlson, C. S.; Weaver, D. S. *Bone* **1997**, *20*, 355-364.
7) Miller, L. M.; Huang, R.; Chance, M. R.; Carlson, C. S. *Synch. Rad. News* **1999**, *12*, 21-27.
8) Miller, L. M.; Tibrewala, J.; Carlson, C. S. *Cell. and Mol. Biol.* **2000**, *in press*.
9) Fogarassy, E.; Slauoi, A.; Fuchs, C.; Regolini, J. L. *Appl. Phys. Lett.* **1987**, *51*, 337.
10) Chyan, O. M. R.; Wu, J.; Chen, J. J. *Appl. Spec.* **1997**, *51*, 1905-1909.
11) Chyan, O. M. R.; Chen, J. J.; Xu, F.; Wu, J. *Anal. Chem.* **1997**, *69*, 2434-2437.